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Where whole-genome duplication is most beneficial: Adaptation of mangroves to a wide salinity range between land and sea

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Abstract

Whole-genome duplication (WGD) is believed to increase the chance of adaptation to a new environment. This conjecture may apply particularly well to new environments that are not only different but also more variable than ancestral habitats. One such prominent environment is the interface between land and sea, which has been invaded by woody plants, collectively referred as mangroves, multiple times. Here, we use two distantly related mangrove species (*Avicennia marina* and *Rhizophora apiculata*) to explore the effects of WGD on the adaptive process. We found that a high proportion of duplicated genes retained after WGD have acquired derived differential expression in response to salt gradient treatment. The WGD duplicates differentially expressed in at least one copy usually (>90%) diverge from their paralogues' expression profiles. Furthermore, both species evolved in parallel to have one paralogue expressed at a high level in both fresh water and hypersaline conditions but at a lower level at medium salinity. The pattern contrasts with the conventional view of monotone increase/decrease as salinity increases. Differentially expressed copies have thus probably acquired a new role in salinity tolerance. Our results indicate that the WGD duplicates may have evolved to function collaboratively in coping with different salinity levels, rather than specializing in the intermediate salinity optimal for mangrove plants. In conclusion, WGD and the retained duplicates appear to be an effective solution for adaptation to new and unstable environments.

KEYWORDS
gene expression, mangrove, neofunctionalization, salt tolerance, whole-genome duplication

1 | INTRODUCTION

Duplication of entire genomic DNA, typically referred to as whole-genome duplication (WGD) or polyploidy, provides rich material for evolution (Ohno, 1970; Conant & Wolfe, 2008; Selmecki et al., 2015; van de Peer et al., 2017). Polyploidy-enabled gene redundancy allows the accumulation of mutations. Genome rearrangements further introduce genetic variation. Polyploids have more genetic variation than diploids, potentially increasing genomic plasticity and the potential to adapt to diverse environmental conditions (Comai, 2005; Leitch & Leitch, 2008; Mable et al., 2011; Hahn et al., 2012; Mattenberger et al., 2017; van de Peer et al., 2009). WGD is particularly common in plants and may play key roles in their survival in diverse environments and during historical environmental changes (Guo et al., 2020; Levin, 1983; Soltis et al., 2009; van de Peer et al., 2009; van de Peer et al., 2017; Zhang et al., 2020).

WGD appears to have occurred in waves through the evolutionary history of plants. Periods of intensive WGD activity coincide with drastic environmental change, such as at the Cretaceous–Palaeogene (K-Pg) boundary (van de Peer et al., 2017; Vanneste et al., 2014). One possibility is that polyploid formation is triggered by environmental stresses (Bretagnolle & Thompson, 1995; Ramsey & Schemske, 1998; van de Peer et al., 2021). For example, increased levels of unreduced or abnormal pollen were observed during major extinction events (Foster & Afonin, 2005; Kürschner et al., 2013). Coincidentally, a higher proportion of polyploids is observed in newly created, disrupted or harsh environments (Diallo et al., 2016; Hahn et al., 2012; Ramsey, 2011; te Beest et al., 2012). On the other hand, a high survival rate of polyploid species during dramatic environmental change can be interpreted to be the result of an adaptive advantage of WGD (van de Peer et al., 2017; Vanneste et al., 2014). A recent wave of WGDs might have contributed to the generation of C_4 from the C_3 photosynthesis system and the expansion of C_4 grasslands in the later Miocene period when the atmospheric CO_2 level was low (Estep et al., 2014; van de Peer et al., 2017). Furthermore, *Arabidopsis thaliana*, rice and citrus polyploids show increased tolerance to environmental stress (Chao et al., 2013; Ruiz et al., 2016; Yang et al., 2014).

Evolutionary benefits of WGD are currently thought to arise because each gene acquires multiple copies, freeing one of them to evolve adaptively in a new environment (Comai, 2005; van de Peer et al., 2009; van de Peer et al., 2017). As described above, this mechanism is remarkably beneficial for organisms adapting from a conventional to a novel environment. However, environmental change does not always result in a stable state. In some cases, organisms must live in a highly dynamic environment where environmental conditions are unstable. In other words, organisms must adapt to a range of environmental conditions rather than a single level. One typical case is the highly variable salinity in intertidal zones that mangrove plants became adapted to. It is of interest to know whether and how WGD has contributed to adaptation to such unstable environments. We hypothesize that coexisting homologous genes may differentiate in function with each copy functioning under separate environmental circumstances, allowing the organism to survive a range of conditions.

Mangrove plants originated from independent inland ancestors and became adapted to intertidal zones where terrestrial and marine habitats meet (Feng et al., 2020; Garcia et al., 2017; He et al., 2020; Liang et al., 2012; Reef & Lovelock, 2015; Tomlinson, 1986; Wang et al., 2016; Xu, He, Zhang, et al., 2017). Under the influence of tidal inundation, rainfall, soil type, topography and many other factors, the environmental salinity of mangrove habitats varies greatly from freshwater to hypersaline conditions in both time and space (Ball, 1998; Ball & Pidsley, 1995; Bunt et al., 1982; Duke et al., 1998; Lin & Sternberg, 1993; Nguyen et al., 2015; Nguyen et al., 2015; Tomlinson, 1986). In some areas salinity fluctuates periodically with tides every day, while at other locations it changes biweekly or even monthly (Lin & Sternberg, 1993; Yang et al., 2013). The difference in rainfall between the dry and rainy seasons causes remarkable seasonal salinity changes (Ball, 2002; Ball & Pidsley, 1995). Salinity also differs among locations. For example, the populations of *Rhizophora apiculata* in Khlong Sawi and Ao Nam Bor in Thailand grow in water with 1‰–2‰ and 35‰ salt, respectively (Kristensen et al., 2017). Salinity also varies greatly from downstream to upstream in estuaries (Yang et al., 2013). Some widely distributed mangrove species maintain normal growth in both terrestrial freshwater conditions and the high salinity of sea water.

A linage-specific WGD event occurred ~70 million years ago in each of the three major mangrove clades that independently originated ~55 million years ago (Figure 1a; He et al., 2020; Xu, He, Zhang, et al., 2017). If both copies of a gene generated by a WGD are retained in the current genome, the homologues may have developed novel functions (neofunctionalization; des Marais & Rausher, 2008; Francino, 2005; Freeling, 2009; Hahn, 2009; Lynch & Conery, 2000; McGrath & Lynch, 2012; Moore & Purugganan, 2005; Ohno, 1970). For example, rapid expression divergence between gene copies has been observed in plants (Blanc & Wolfe, 2004; Ha et al., 2009; Wang et al., 2012). Hence, we examined expression profiles of genes with multiple copies retained from WGD (hereafter we call these genes "WGD-duplicates") under different salinity concentrations, using *R. apiculata* and *Avicennia marina* as model systems. Using available whole genome sequences (He et al., 2020; Xu, He, Zhang, et al., 2017), we compared the evolution of expression profiles to genome sequence divergence.

2 | MATERIALS AND METHODS

2.1 | Experimental design for salt treatment

To explore the role of WGD retentions in adaptation to different salinity levels, we designed an experiment to treat seedlings of two mangrove species with varying salt concentrations. For each mangrove species, 18 seedlings normally grown in 250–340 mM NaCl were collected from Hainan, China, and cultivated in a glasshouse. The seedlings were transferred to sandy soil and cultured using ½ Hoagland's solution for 3 weeks to help the plants restore normal growth. For each species, the seedlings were divided into three groups, and each group was treated with one salt concentration. To minimize gene expression variation among individual plants, each group contained two biological replicates, with three individual seedlings pooled within each replicate.

To fully cover the range of salinity and the growth status of the two mangrove species, the three experimental groups were

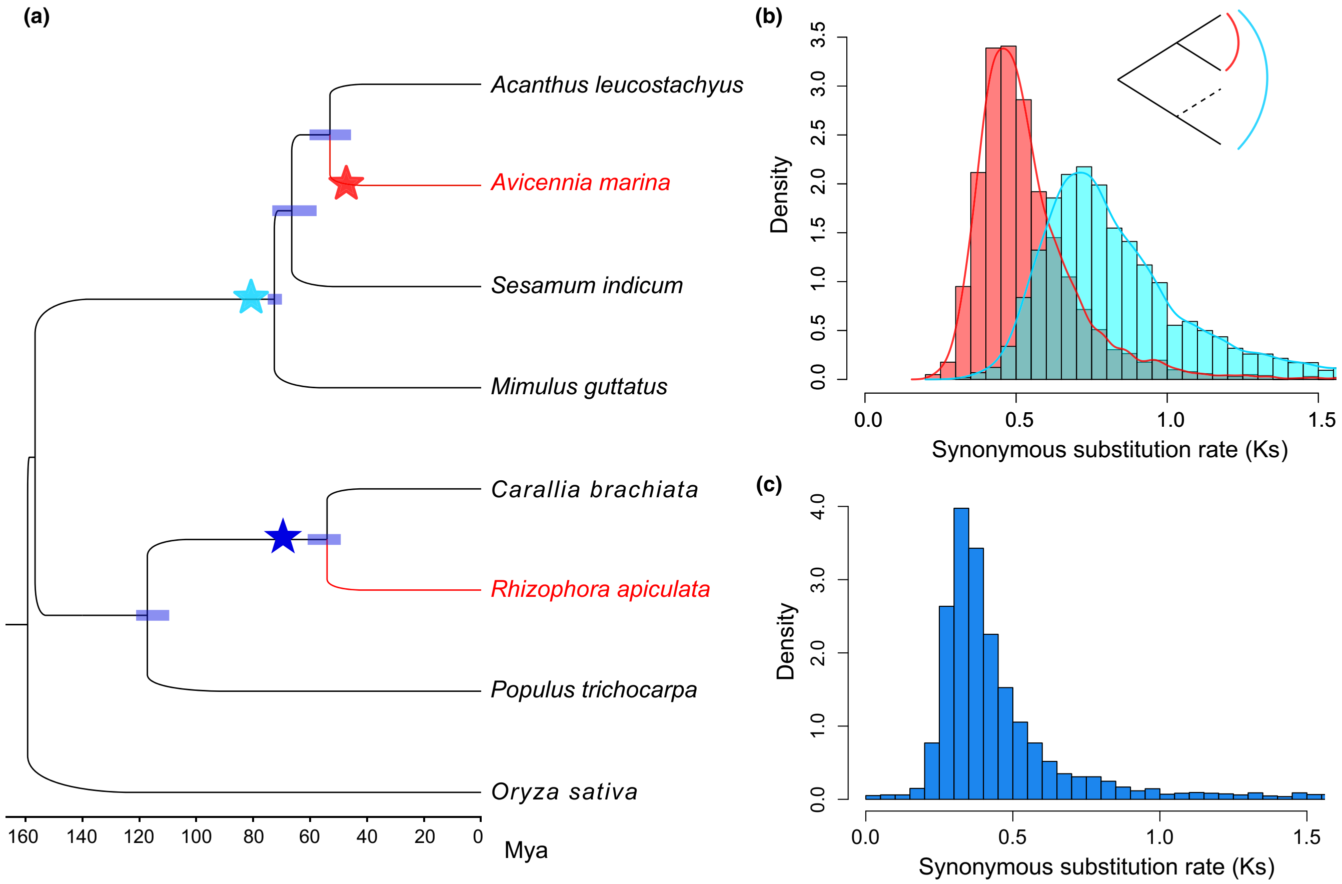


FIGURE 1 Identification of WGD events and gene duplicates retained after WGD in *Rhizophora apiculata* and *Avicennia marina*. (a) Phylogeny of the two mangrove species and WGD events. Reanalysis of the *A. marina* genome revealed two recent WGD events. The WGD events are marked with stars. The relatively old WGD event was shared with Laminales. The younger event occurred after the divergence between *Avicennia* and *Acanthus*. Dating of the WGD events relies on sequence divergence between and within species (Figure S4). The phylogeny and divergence time were inferred in our previous study using codon sequences of single-copy genes with fossil records for recalibration (He et al., 2020). The blue bars show 95% confidence intervals. (b) Distribution of Ks (synonymous substitution rate) values between homologous gene pairs produced by WGD events in *A. marina*. Homologous groups with three or four copies were used for the calculation. Within each group, gene pairs were classified as produced by the older (light blue) or the younger (red) WGD event. (c) Distribution of Ks values between homologous gene pairs produced by WGD events in *R. apiculata*

irrigated using solutions containing 0, 250 or 500 mM NaCl for 7 days. The time and salinity range of the treatment were set based on previous studies (Burchett et al., 1989; Lechthaler et al., 2016; Liang et al., 2012; Natarajan et al., 2021; Nguyen et al., 2015; Wang et al., 2016; Wang et al., 2016; Zhu et al., 2011). Both mangrove species can grow at varying salinities, with growth most vigorous at intermediate levels of ~50% seawater (~250 mM NaCl; Burchett et al., 1989; Clough, 1984; Kristensen et al., 2017; Nguyen et al., 2015; Patel et al., 2010; Tomlinson, 1986). In both freshwater and at higher salinity (~500 mM NaCl), vigour is lower and similar. A 250 mM NaCl concentration is approximately equal to the optimal environmental salinity. The 0 and 500 mM conditions represent fresh water of the inland environment and normal sea water salinity (lower and upper limits), respectively. To eliminate short-term salinity shocks and identify genes regulated by long-term adaptation to a wide salinity range, the salt treatments were conducted for 7 days.

2.2 | RNA sequencing

After treatment for 7 days, healthy young leaves and roots of each seedling were sampled and immediately frozen in liquid nitrogen and stored at −80°C. Leaves and roots play key roles in the salinity response of plants, including the two mangrove species. Roots act primarily in salt sensing, uptake and translocation (Chandna et al., 2014; Gong et al., 2020; Rasool et al., 2013). Both *Rhizophora apiculata* and *Avicennia marina* have evolved to block salt entry into plants (Ball, 1988; Parida & Jha, 2010). Maintenance of photosynthesis in leaves also plays a key role in salinity tolerance (Jajoo, 2013). The vacuoles of leaf cells also allow Na^+ accumulation and storage, facilitating osmotic adjustment and plant growth (Gong et al., 2020). These mechanisms are important in *R. apiculata*. *A. marina* has evolved salt glands to secrete salt from leaves (Parida & Jha, 2010).

Three samples from the same group were pooled within a biological replicate to minimize variation among individual plants. Total

RNA was extracted using a Plant RNA Kit (Omega Bio-Tek). mRNA library construction and sequencing were performed by Novogene. mRNA libraries were constructed using reagents provided in the Illumina TruSeq RNA Sample Preparation Kit. Poly-A-containing mRNA molecules were first isolated using poly-T oligo-attached magnetic beads. Then mRNA molecules were fragmented and copied to first-strand cDNA using reverse transcriptase and random hexamer primers. The second cDNA strand was synthesized using DNA Polymerase I and RNase H. After the end repair process, the cDNA fragments were ligated with adaptors, purified and enriched via the polymerase chain reaction (PCR). After passing quality controls, cDNA molecules were clustered and sequenced on the Illumina HiSeq 2000 platform. Raw sequence reads were first cleaned by trimming adaptor sequences. Sequencing errors were minimized by removing low-quality read pairs. Low-quality read pairs were discarded if they met one of the following criteria: (i) contained more than 5% unknown bases (N); (ii) contained more than 20% of bases with quality ≤10; or (iii) had an average base quality less than 20. The last base of the filtered reads was also trimmed according to FASTQC results (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/).

2.3 | Identification of differentially expressed genes responsive to salt treatment

Gene expression regulation is the primary driver of adaptation to environmental fluctuation (Kenkel & Matz, 2017; López-Maury et al., 2008). We therefore quantified gene expression levels and identified differentially expressed genes (DEGs) from RNA sequencing data following a standard pipeline. Clean reads from each sample were aligned to the *R. apiculata* or *A. marina* genome using TOPHAT version 2.1.1 (Kim et al., 2013) and BOWTIE2 version 2.3.4.3 (Langmead & Salzberg, 2012). Genome-guided transcriptome assembly and differential transcript expression analyses were then performed using CUFFLINKS version 2.2.1 (Trapnell et al., 2012). Expression levels were reported as fragments per kilobase of exon model per million mapped reads (FPKM). DEGs were identified from three comparisons: 250 mM NaCl versus 0 mM NaCl, 500 mM NaCl versus 250 mM NaCl, and 500 mM NaCl versus 0 mM NaCl. Transcripts with *q*-values (false discovery rate [FDR]-adjusted *p*-values) below 5% and a fold change greater than two in either comparison were considered as differentially expressed (DEGs). A gene is "upregulated" if the expression increased with increasing salt concentration and "downregulated" if its expression decreased at high salt. The remaining genes are denoted as having "no significant difference."

2.4 | Identification of WGD-duplicates

R. apiculata and *A. marina* protein sequences were obtained from our previous studies (Figure 1a; He et al., 2020; Xu, He, Zhang, et al., 2017). Both mangrove species are diploids and were reported to have experienced lineage-specific WGD events. WGDs produce collinear blocks with a linear arrangement of homologous genes. Using this principle, we identified WGD-duplicates by employing MCSCANX (Wang, Tang, et al., 2012). Specifically, collinear blocks were defined as regions of the genome that harboured at least five genes with homologues elsewhere in the genome and in the same order. To obtain homologous genes within species, each protein-coding gene was compared to all other genes in the same genome using BLASTP with an e-value cut-off of $<10^{-5}$ and identity score ≥40%. Homologous gene pairs within collinear blocks were identified as candidate WGD-duplicates.

These candidate WGD-duplicates also contain orthologues produced by more ancient WGD events (Jiao et al., 2011; van de Peer et al., 2017). To focus on the recent lineage-specific WGD events in this study, we filtered out WGD-duplicates produced by ancient WGD events using a pairwise synonymous substitution rate (Ks) cutoff. Protein sequences of gene pairs were first aligned individually using MUSCLE (Edgar, 2004) and converted to codon alignments with PAL2NAL (Suyama et al., 2006). After removing bad alignments using GBLOCKS (Talavera & Castresana, 2007) and alignments shorter than 50 codons, Ks was calculated for each gene pair using KAKS_CALCULATOR 2.0 with the YN model (Zhang et al., 2006). We then identified collinear blocks produced by recent WGDs by examining the median Ks within collinear blocks (Figure S1). Median Ks within collinear blocks can be clearly distinguished, consistent with the recent and ancient WGD events. In *R. apiculata*, collinear blocks with median Ks <1.00 were retained as the products of the recent WGD event. In *A. marina*, collinear blocks with median Ks, 1.50 were retained. To filter blocks potentially produced by recent segmental duplications, we also removed two blocks with median Ks < 0.05 in *R. apiculata*. Gene pairs in the retained blocks with Ks > 1.8 were also removed. The genome distribution of the collinear blocks was visualized using MCSCANX software.

We identified two recent WGD events in *A. marina* based on the bimodal Ks distribution and the existence of quaternary collinear blocks. Ks distance was used to reconstruct a phylogeny within homologous groups. Because of frequent gene loss after WGD, these homologous groups after two rounds of WGD may have four, three or two homologues. The quaternary (ternary) homologous groups are expected to have two (one) gene pairs that were produced in the recent WGD event with low Ks values and four (two) gene pairs with larger Ks values. Pairwise Ks values were first calculated within a homologous group according to the procedure above, and genes were then grouped by their Ks values.

To make a more detailed comparison, non-WGD-duplicates were classified into singletons, dispersed duplications and tandem duplications based on the criteria of Wang, Tang, et al. (2012). Homologous gene copies that are near each other (no other gene between them) or separated by no more than 10 genes were classified as tandem duplications. Homologous gene copies that are not WGD-duplicates or tandem duplications were classified as dispersed duplications. Genes without homologues were classified as singletons.

2.5 | Expression profiles of WGD-duplicates under different salinity levels

If many duplicated genes produced by WGDs help mangroves cope with different salinity levels, we expect to observe an elevation of differential expression in WGD-duplicates. We examined the proportion of DEGs among WGD-duplications and the rest of the genome in leaves and roots. In *A. marina*, the analyses were performed separately for doublet, triplet and quadruplet homologous groups.

Mangroves are well adapted to a wide salinity range in intertidal zones. Thus, we tested whether salt-regulated genes change their expression during adaptation to different salt concentrations. We explored combinations of expression changes from 0 to 250 or 250 to 500 mM, with "−1," "0" and "1" representing downregulation, unchanged expression and upregulation, respectively. Hence, we have eight combinations showing expression change in at least one comparison (Figure 2b), excluding "0;0" which indicates constant expression regardless of salt concentration. For example, the combination "−1;1" indicates downregulation from 0 to 250 mM followed by upregulation from 250 to 500 mM.

We then tested whether any differential expression combination contains more genes than expected. To determine which clusters have statistically significant enrichment in the number of genes assigned, we used a permutation test with 10,000 runs. The permutation test was performed by randomly shuffling gene expression levels under three salt conditions and creating an artificial expression profile. In each run, the frequency of artificial expression combinations was compared with the real frequency. After 10,000 runs, the frequency of runs with no fewer genes within a combination than the real observation was used as a p-value for significance tests. For example, if only one of the 10,000 runs has more genes in the combination "−1;1" than the observed number, the p-value = 1/10,000. We then tested the enrichment of expressional combinations in WGD-duplicates relative to non-WGD-duplicates using Fisher's exact test.

2.6 | Assessment of expression neofunctionalization among WGD retentions

We consider duplicated genes with both copies showing identical expression combinations across salinity levels to have retained ancestral function (functional conservation; Figure 3a). Duplicates showing inconsistent expression combinations are candidates for neofunctionalization. For example, the primary case of neofunctionalization can be simply inferred if one copy of the duplicate differs in expression from the other copy in leaves (Figure 3a). If the two copies show different expression combinations in both leaves and roots, we infer two neofunctionalization events (Figure 3a). Groups with unchanged expression of both copies under salinity level changes (with the combination "0;0") were identified as functionally conserved. Groups with at least one copy showing differential expression under salinity level changes are candidates for salt-regulated gene groups and were used for subsequent analyses.

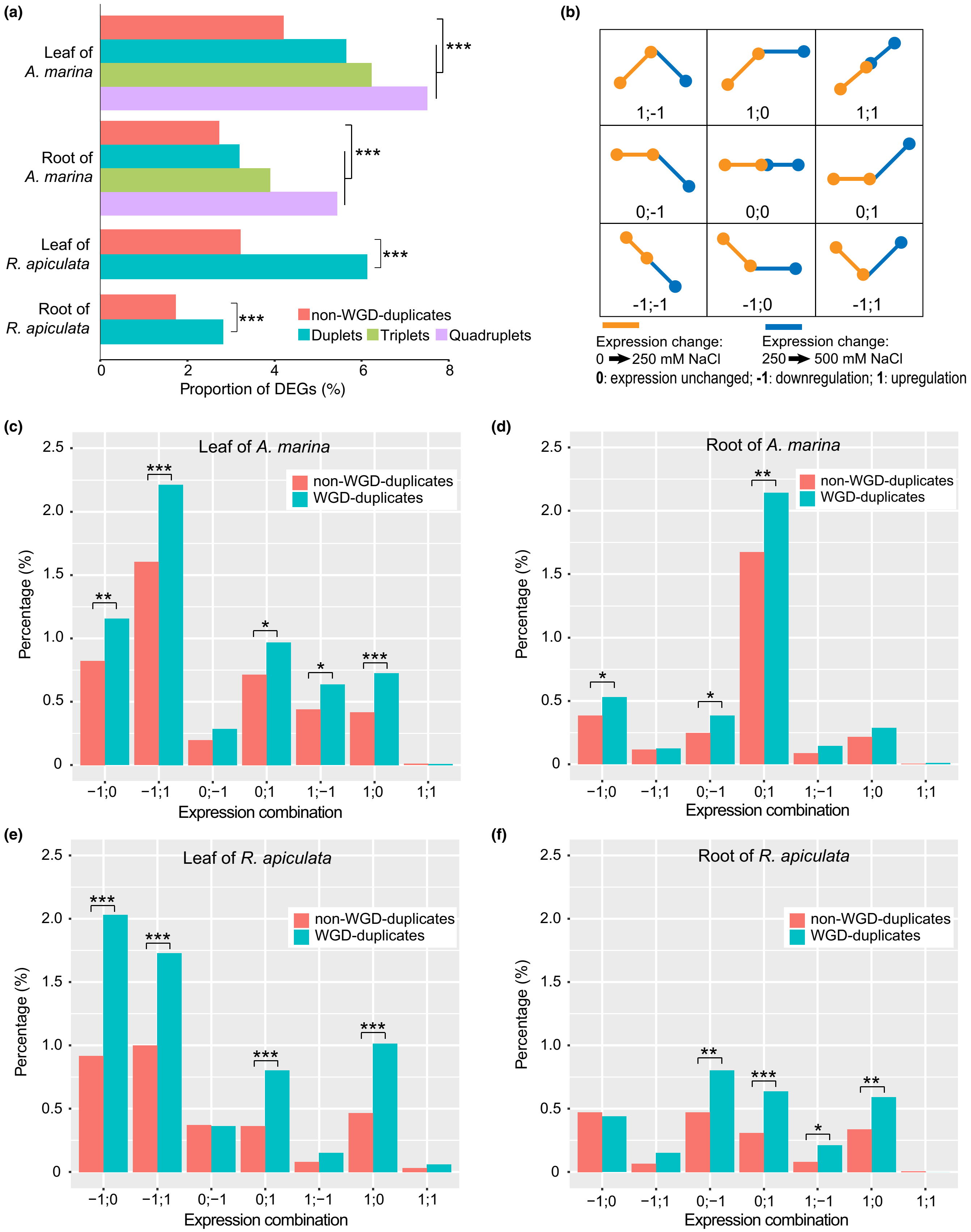
The two lineage-specific WGDs in *A. marina* produced triplet or quadruplet homologous groups, which were used to infer whether differential expression combinations are derived from the ancestral state of stable expression. If one copy of a triplet or quadruplet homologous group shows a differential expression combination but the other two or three copies are expressed invariantly, the most parsimonious explanation is that the differential expression is derived. Conversely, if one copy expressed at the same level regardless of salt concentration but the other two or three copies show an identical differential expression combination, we infer that constant expression is the derived state.

Doublet expression states (i.e., gene groups with two homologues) were inferred using the other mangrove species as the outgroup. We first identified orthologous groups between *R. apiculata* and *A. marina* using BLASTP, with an e-value cut-off of 1.0×10^{-10} and an identity score ≥40%. The two copies within a group can be assigned the same or different orthologues in the outgroup species. The groups with both copies having the same orthologue in the outgroup species or the two orthologues of the two copies having the same expression state in the outgroup species can be used for expression state inference. If the outgroup and one copy of the target group have the same state of differential expression, then the other copy with constant expression is the derived state, otherwise the differential expression is inferred as the derived state.

2.7 | Functional enrichment analysis

Gene Ontology (GO) term annotation and pathway annotation were performed using homologous genes in *Arabidopsis thaliana*. For each mangrove species, we identified *A. thaliana* homologues using BLASTP, with a e-value cut-off of 1.0×10^{-10} and an identity score ≥40%. *Arabidopsis thaliana* GO annotation information was obtained from TAIR10. For each class of neofunctionalized retained genes, GO enrichment analysis was performed using the agriGO version 2.0 platform (Tian et al., 2017) with the singular enrichment analysis tool. Fisher's exact tests and Yekutieli's FDR under dependency were used to identify significantly enriched GO terms ($p < .05$ and $Q < .05$).

FIGURE 2 Overrepresentation of differentially expressed genes among WGD-duplicates. (a) WGD-duplicates are overrepresented among salt-responsive genes in roots and leaves of both species. In *Avicennia marina*, this overrepresentation is higher for homologue groups with more copies. The bars represent proportions of differentially expressed genes (DEGs) in non-WGD-duplicates or WGD-duplicates (including duplets, triplets and quadruplets). (b) Eight possible differential expression patterns as salt concentrations increase from 0 to 250 mM and from 250 to 500 mM. Numbers "1," "0" and "−1" represent upregulation, unchanged expression and downregulation, respectively. (c–f) Each red bar represents what percentage of all non-WGD-duplicates could be classified into the specific expression combination, while the blue bars represent that of all WGD-duplicate genes. Panels (c)–(f) show the comparison in leaves (c) and roots (d) in *A. marina* and in leaves (e) and roots (f) in *Rhizophora apiculata*. No genes were classified as having the pattern "−1;−1". p-values were calculated by Fisher's exact test. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$


(a)
Leaf of *A. marina*
Root of *A. marina*
Leaf of *R. apiculata*
Root of *R. apiculata*
***
***
***
***
non-WGD-duplicates
Duplets
Triplets
Quadruplets
0
2
4
6
8
Proportion of DEGs (%)
(b)
1;-1
1;0
1;1
0;-1
0;0
0;1
-1;-1
-1;0
-1;1
Expression change: 0 ➔ 250 mM NaCl
Expression change: 250 ➔ 500 mM NaCl
0: expression unchanged; **-1**: downregulation; **1**: upregulation
(c)
Leaf of *A. marina*
(d)
Root of *A. marina*
(e)
Leaf of *R. apiculata*
(f)
Root of *R. apiculata*
non-WGD-duplicates
WGD-duplicates
Percentage (%)
0
0.5
1.0
1.5
2.0
2.5
−1;0
−1;1
0;−1
0;1
1;−1
1;0
1;1
Expression combination
*
**
***

Arabidopsis thaliana pathway annotations were downloaded from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. To confirm the results of GO enrichment analysis, we used an alternative approach to perform GO term annotation in both mangrove species using the EGGNOG-MAPPER (Huerta-Cepas et al., 2017). Enrichment analyses were then performed using the R package clusterProfiler (Yu et al., 2012). Fisher's exact tests and Bonferroni correction were used to identify significantly enriched GO terms ($p < .05$ and $Q < .05$).

3 | RESULTS

3.1 | Identification of gene pairs retained after WGDs

We identified 4287 pairs of gene duplicates produced from the sole recent WGD in *Rhizophora apiculata* (Figure 1c and Figure S2). *Avicennia marina* probably experienced two recent WGD events, so we identified homologous groups with three or four copies within collinear blocks, excluding blocks originating from more ancient WGD events. These multicopy homologues produce a bimodal (at 0.5 and 0.75) distribution of pairwise synonymous substitution rates (Ks; Figure 1b). Furthermore, quaternary and ternary collinear block groups were observed (Figure S3). All these observations clearly indicate that two lineage-specific WGDs had occurred in the genome of *A. marina*. By comparing between- and within-species Ks values, we roughly dated the two WGD events (Figure 1a; Figure S4) at before the diversification of Laminales and after the divergence between the genera *Avicennia* and the *Acanthus*. Based on Ks-distance, we reconstructed phylogenies within each quaternary/ternary homologue group and identified 6013 groups in *A. marina*, including 4353 doublets, 1278 triplets and 382 quadruplets (Figure S5).

3.2 | Salt-regulated transcripts are overrepresented among gene duplicates retained after WGD

3.2.1 | Overrepresentation of differentially expressed genes among WGD-duplicates

The seedlings of *R. apiculata* and *A. marina* were treated with 0, 250 or 500 mM NaCl, with 250 mM simulating a mix of freshwater and seawater in intertidal zones, 0 mM simulating fresh water and 500 mM NaCl full sea water. RNA sequencing was used to quantify the gene expression levels (Tables S1–S3; Figure S6). DEGs were identified by comparing expression at 0–250 mM and 250–500 mM (Tables S4 and S5).

DEGs are significantly overrepresented among WGD-duplicates in both species. In leaves and roots of *R. apiculata*, 6.1% and 2.8% (403 and 186 of 6557) of WGD-duplicates are DEGs, significantly higher than the 3.2% and 1.7% (479 and 259 of 14,813) of non-WGD-duplicates (all genes except WGD-duplicates; Figure 2a; $p < 2.2 \times 10^{-16}$ and $p = 3.57 \times 10^{-7}$; Fisher's exact test). Similarly, these proportions increased from 4.2% and 2.7% (766 and 497 of 18,212) to 6.0% and 3.6% (668 and 403 of 11,152) in *A. marina* (Figure 2a; $p = 5.93 \times 10^{-12}$ and $p = 1.36 \times 10^{-5}$; Fisher's exact test). These increases are even more striking among triplets and quadruplets of *A. marina* (Figure 2a). We then classified the non-WGD-duplicates into singletons, dispersed duplications and tandem duplications. We found both the number and the proportion of DEGs among WGD-duplications are much higher than among singletons and dispersed duplications (Tables S6 and S7). Although DEG proportions are slightly higher among tandem duplications, the numbers of DEGs among WGD-duplications is much higher.

To further confirm the relationship between WGD-duplicates and salt change tolerance, we examined whether WGD-duplicates contained more known salt tolerance-related genes than expected based on the genome average. We consider *Arabidopsis thaliana* genes annotated as “response to salt stress” and/or “response to osmotic stress” (TAIR10) to be “known salt tolerance related genes.” These two terms are widely used in identifying genes underlying salt tolerance (Li et al., 2008; Miyama & Tada, 2008; Yao et al., 2011). We identified 21,077 orthologous groups between *Arabidopsis thaliana* and *R. apiculata*, with 2.9% (618/21077) annotated as salt-related genes. Among WGD retentions, the proportion is 5.7% (230/4054 gene pairs; Figure S7), significantly higher than the whole-genome expectation ($p < 2.2 \times 10^{-16}$, Fisher's exact test). The overrepresentation is also significant in *A. marina* (309/5612 vs. 618/20,542; $p < 2.2 \times 10^{-16}$; Figure S7). These results clearly indicate that WGD-duplicates play important roles in adaptation of these mangrove species to different salinity levels.

3.2.2 | WGD-duplicates show higher expression under both lower and higher salt concentrations

We then classified the DEGs into eight groups according to the direction of their transcription level change in the 0/250 and 250/500 mM NaCl comparisons. Transcripts can be upregulated (1), downregulated (−1) or not significantly changed (0). These three expression categories lead to nine combinations between the two salinity level comparisons. We exclude the uninformative “0;0” category that contains transcripts that never change their levels, leaving eight combinations. No genes fall into the “−1;−1” combination and is not shown. Recent WGD-duplicates are more numerous than genes from the rest of the genome among the categories with differentially expressed transcripts, especially in the combinations with many DEGs (Figure 2c–f).

Expression patterns in leaves of both species are quite similar, with four of the eight combinations containing significantly more WGD-duplicates than non-WGD-duplicates (Figure 2c,e; $p < .05$, Fisher's exact test). The combinations include “0;1” and “1;0,” where gene expression monotonously rises with increasing salinity. Surprisingly, we found that the most abundant combinations in both mangrove taxa were “−1;0” and “−1;1.” These two combinations

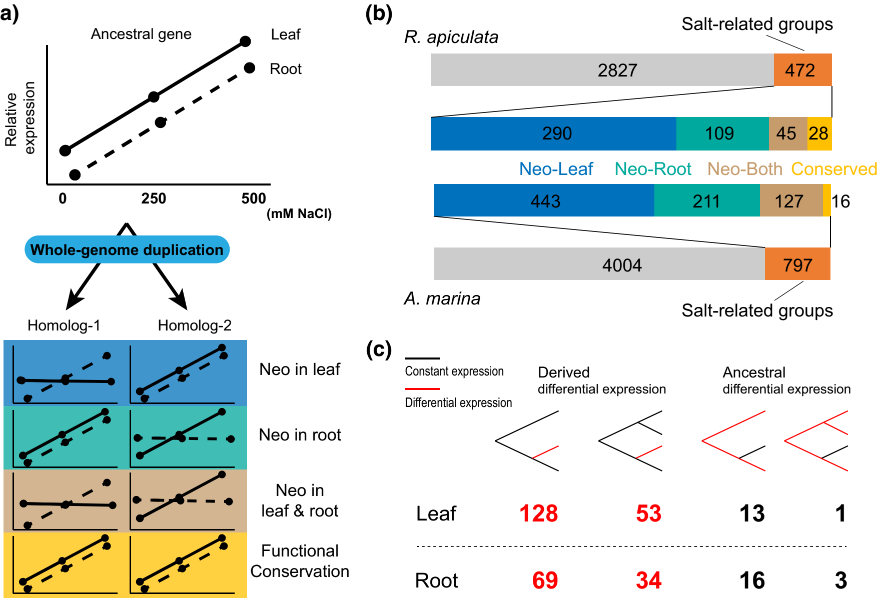


FIGURE 3 Identification and quantification of neofunctionalized regulatory salt-responsive WGD-duplicates. (a) Criteria for identifying the fates of WGD-duplicates. Gene pairs with expression patterns affected by salt treatment in the same way, in both leaves and roots, were classified as functionally conserved. Gene pairs for which at least one copy was affected by salt treatment and the two copies had different expression patterns were classified as neofunctionalized. (b) Classification of homologous groups according to the criteria shown in (a). Numbers represent groups that have expression profiles consistent with the labelled category. In both mangrove taxa, most homologous groups had neofunctionalized expression patterns under salt changes. (c) Inference of derived expression state using triplet and quadruplet homologue groups in *Avicennia marina*. When a triplet or quadruplet homologous group has only one copy differentially expressed and other copies constantly expressed, the most parsimonious explanation is that differential expression is the derived state since genome duplication. Similarly, when only one copy is constantly expressed and other copies share the same expression pattern, constant expression is inferred as the derived state. In both leaves and roots, differential expression is derived

describe a significantly larger number of DEGs than expected from the genome average ($p < .0001$; permutation test). This enrichment is significantly enhanced among WGD-duplicates (Figure 2c,e; $p < .001$; Fisher's exact test). The combination "−1;1" is especially informative, as it implies that genes are expressed at higher levels in both fresh and hypersaline sea water than in medium-salinity water (Figure 2b). This observation reinforces the view that medium salinity is optimal for mangrove plants. Notably, the combination "1;−1" was also significantly enriched in WGD-duplicates in *A. marina* (Figure 2c; $p = .015$, Fisher's exact test), implying that gene expression is downregulated in both fresh and hypersaline water.

In both mangrove species, the number of DEGs in roots was significantly smaller than in leaves ($p < 2.2 \times 10^{-16}$, Pearson's Chi-squared test). In *A. marina*, only the combination "0;1" had a significantly higher number of DEGs than the other combinations ($p < .0001$; permutation test), and the enrichment was enhanced among WGD-duplicates ($p = .003$, Fisher's exact test). Genes showing this combination were upregulated only when the salinity increased from medium to high. In *R. apiculata*, all combinations had a small number of DEGs in roots, although the combinations "0;−1," "1;−1," "0;1" and "1;0" were significantly enriched in DEGs among the WGD-duplicates ($p < .05$, Fisher's exact test).

To further confirm the results, we examined the differential expression between 0 and 500 mM NaCl treatment. We identified 382 and 234 DEGs in *R. apiculata* leaves and roots, and 108 and 788 DEGs in *A. marina* leaves and roots. We expect the expression at 0 and 500 mM to be similar for genes in the "−1;1" category. As expected, we found only 6.7% (18/268) and 2.1% (12/567) of the leaf "−1;1" genes to be differentially expressed in the 0–500 comparison in leaves of *R. apiculata* and *A. marina*, respectively. These results support the hypothesis that medium salinity is optimal for mangrove plants, and both fresh and hypersaline sea water induce similar stresses.

3.3 | Neofunctionalization of WGD-duplicates

It is clear that WGD-duplicates contribute to mangrove adaptation to wide salinity ranges from fresh to full seawater as the tides change. We therefore wanted to know how genes evolved during these adaptive events. We examined expression of duplicated gene pairs to test whether neofunctionalization had occurred. Among the 4801 expressed homologous gene groups of *A. marina* and 3299 in *R. apiculata*, we identified 797 (16.6%) and 472 (14.3%) candidates for salt change-related WGD-duplicates. For *A. marina*, 443 (55.6%) of the 797 groups diverged in expression only in leaves, 211 (26.5%) only in roots and 127 (15.9%) in both tissues. Only 16 (2.0%) homologous groups were functionally conserved (Figure 3b). The observations in *R. apiculata* are similar: only 28 (5.9%) groups were functionally conserved, while the remaining 444 (94.1%) groups diverged in expression in leaves (290, 61.4%), roots (109, 23.1%) or both tissues (45, 9.5%). This expression divergence implies a high frequency of neofunctionalization among WGD-duplicates and indicates that this process played a primary role in mitigating the effects of salinity change. Similar observations have been reported in *Arabidopsis thaliana*, maize, teleost fish and *Drosophila*, where most gene duplications have diverged in expression, implying a change in function (Assis & Bachtrog, 2013; Blanc & Wolfe, 2004; Hughes et al., 2014; Lien et al., 2016).

The most common divergence pattern involves one copy of a duplicate pair changing its expression in response to at least some salt level changes, while the other remains stably expressed under all conditions (combination "0;0"; Figure S8). However, it is not enough to identify duplicate pairs that show expression pattern divergence. We also must determine whether stable expression is ancestral and whether the salt-responsive expression is derived. Using a maximum-parsimony method, we find that differential expression is much more likely to be the derived state (Figure 3c). We inferred that 181 homologous groups in leaves and 103 in roots show derived salt-responsive expression, while only 14 and 19 groups appear to show derived stable expression in leaves and roots, respectively. Using the other mangrove species as the outgroup, many cases of derived expression changes were also observed for doublet groups in both species (Table S8). Thus, it appears that mangrove gene duplicates that have been retained after the recent WGD have evolved salt-responsive expression from constant expression to respond to their new variable saline environment.

3.4 | Neofunctionalization of WGD-duplicates contributed to adaptation to varying salinity levels

To elucidate how duplicate gene neofunctionalization may have contributed to adaptation to variable salinity levels, we further examined the function and sequence evolution of the neofunctionalized genes. To increase power, we focused on the expression combination "−1;1" in leaves, which was the most abundant in *A. marina* and the second most abundant in *R. apiculata*. The GO functional annotation was conducted using two approaches, with consistent results (Figure 4a; Tables S9–S12 and Section 2). The following descriptions are based on searching for homologues in *Arabidopsis thaliana*.

In *A. marina*, the 209 duplicated gene groups with differential expression combination "−1;1" are more likely than expected by chance to be annotated as contributors to stress response (Figure 4a; Table S10; FDR < 0.05): 91 of them are labelled with the GO term "response to stimulus." For example, the gene *HKT1* (high-affinity K^+ transporter 1) has been reported to play key roles in adaptation to high salinity (Busoms et al., 2018; Feng et al., 2021; Oh et al., 2014; Rus et al., 2006; Wu et al., 2012). One *HKT1* copy shows the expression pattern "−1;1" in leaves, while other copies are stably expressed across salinity levels. *SOS6* encodes a cellulose synthase-like protein and plays a critical role in osmotic stress tolerance, as its mutant plants are hypersensitive to salt stress (Zhu et al., 2010). This gene is probably involved in regulating reactive oxygen species (ROS) under stress. Another gene, *ITN1*, encoding an ankyrin repeat protein, affects the abscisic acid (ABA)-mediated production of ROS and is involved in salt stress tolerance (Sakamoto et al., 2008). At the pathway level, we found that the "plant hormone signal transduction" pathway was significantly overrepresented ($p = .0005$, Fisher's exact test; FDR = 0.005). Seven of the "−1;1" homologous groups were annotated as belonging to this pathway, with three genes in jasmonic acid signalling and one gene each in auxin, cytokinin, gibberellin and ethylene signalling. Differential expression of the key regulators of plant hormone signal transduction may play key roles in both developmental processes and stress response. Similar differential expression of genes involved in plant hormone signalling was also observed in a closely related species, *Avicennia officinalis* (Krishnamurthy et al., 2017).

In *R. apiculata*, the "−1;1" gene pairs were significantly enriched for "extracellular region," "carbohydrate metabolic process," "cellular developmental process" and similar terms (Figure 4a; Table S9; FDR < 0.05). With 31 of the 102 "−1;1" gene pairs assigned to the term "extracellular region," the enrichment was significant. All genes associated with the term "carbohydrate metabolic process" are involved in cell wall organization. Genes located in the extracellular region are sensitive to and the first to be affected by salt concentration changes (Feng et al., 2016; le Gall et al., 2015; Liu et al., 2015; Zagorchev et al., 2014). Our previous study in mangrove plants revealed significant amino acid composition changes in extracellular proteins (He et al., 2020). These observations indicate that extracellular proteins are mainly adapted to medium salinity and are influenced by both fresh and hypersaline water. Genes encoding extracellular or membrane proteins are essential for normal plant growth under stressful conditions. The *CESA1* and *SUS4* genes encode cellulose synthase and help maintain normal plant growth under stressful conditions (Endler et al., 2015; Kang et al., 2008). Several genes in the pectin biosynthesis pathway also show the "−1;1" expression pattern. Two homologous groups encoding aquaporins help maintain osmotic equilibrium (Maurel et al., 2008). The *VIN* gene encodes vascular invertase and controls stomatal aperture and cell expansion (Chen et al., 2016; Wang et al., 2010). Another gene, *ROPGEF1*, negatively regulates the ABA signalling pathway and stomatal closure (Li & Liu, 2012).

Overall, the two mangrove species have recruited distinct genes in dealing with salinity level changes despite convergently derived expression profiles. There are only six WGD-duplicate groups in common between the two species in the "−1;1" combination. Although the WGD-duplicates were enriched in transcription factors in both mangrove species, no overlap of the transcription factors was found (Figure 4a). In *R. apiculata*, 15 "−1;1" gene pairs were annotated with "transcription factor activity." An R2R3-MYB transcription factor controls trichome maturation and cuticle formation (Camoirano et al., 2020). Another MYB transcription factor, PFG3 (Production of Flavonol Glycosides 3), positively regulates flavonol biosynthesis (Pandey et al., 2014). ERF041, a member of the DREB subfamily A-4 of the ERF/AP2 transcription factor family, promotes

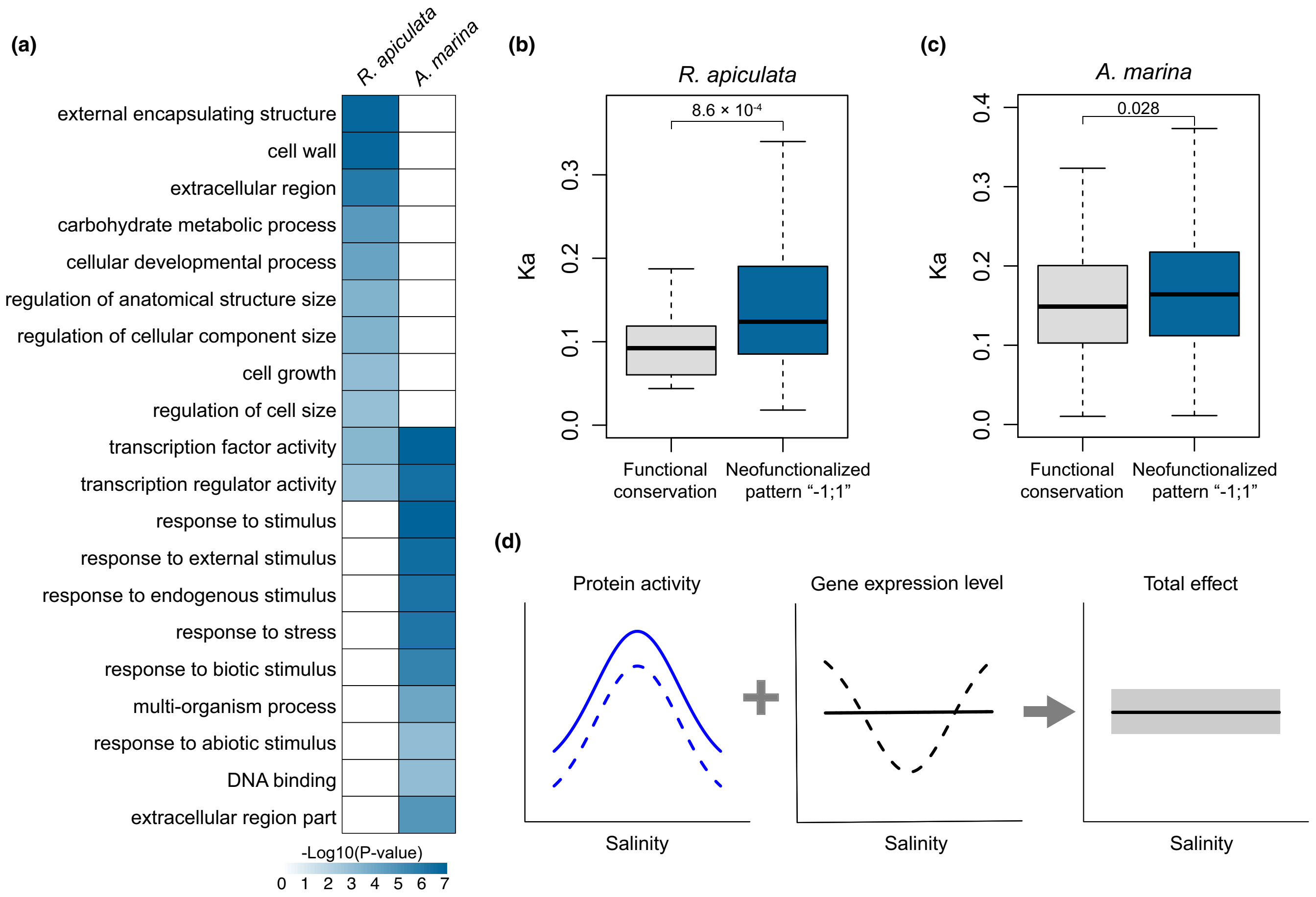


FIGURE 4 Functional categories and rapid sequence evolution of the neofunctionalized "−1;1" expression pattern of WGD-duplicates. (a) GO term enrichment of the expression combination "−1;1" in WGD-duplicates of *Rhizophora apiculata* and *Avicennia marina*. For both species, orthologous relationships with *Arabidopsis thaliana* were determined using the bidirectional best hit method. *Arabidopsis thaliana* GO terms were then assigned to the corresponding orthologues in the mangroves. Non-WGD-duplicates were used as the background for enrichment analysis. *p*-Values were calculated using Fisher's exact test. GO terms with FDR <0.05 are shown. (b,c) Comparison of nonsynonymous substitution rates (Ka) between neofunctionalized "−1;1" gene pairs and salt-related gene pairs with conserved expression in *R. apiculata* (b) and *A. marina* (c). *p*-Values were calculated using Student's *t* test. (d) Hypothesized mechanisms underlying the influence of gene retention on tolerance of salinity changes in mangroves. For both mangrove taxa, the most abundant DEGs were induced by both hypotonic and hypersaline conditions. This phenomenon may indicate that mangrove cellular processes have adapted to medium salinity and that both low and high salinity are stressful for the plants. Extensive amino acid composition changes in mangrove species also support this hypothesis (He et al., 2020). This figure depicts a possible role of this class of genes in the different salinity level tolerance of mangroves. The solid lines show ancestral and the dashed lines derived states. The left panel shows possible changes in protein activity of developmental genes with salinity change in mangroves. We hypothesize that the optimal condition for mangrove protein function is 250 mM NaCl. The middle panel shows the expression level change of homologous gene pairs as salt levels change, as found in the above analyses. According to the activity and expression pattern of homologous gene pairs, we hypothesize in the right panel that the combined effects of the two copies should be stable across salt concentrations

primary cell wall formation, and upregulates *CESA* genes (Nakata et al., 2021). In *A. marina*, 32 gene pairs with combination "−1;1" were annotated with "transcription factor activity." Seven of the transcription factors are involved in phytohormone signalling, such as auxin, gibberellic acid, ethylene and abscisic acid. This indicates the importance of phytohormones to the response to salinity level changes in this species.

It has been proposed that relaxed purifying selection due to gene redundancy and subsequent positive selection contribute to gene neofunctionalization. The former process allows the accumulation of mutations, while the latter rapidly fixes beneficial mutations. Both processes accelerate sequence evolutionary rates (Comai, 2005; van de Peer et al., 2017). We therefore tested the correlation between expression profile divergence and evolutionary rate in retained duplicate genes by comparing nonsynonymous substitution rates (Ka) in duplicates that changed expression profiles to the controls that retained the same expression pattern in response to salt treatment. We found significantly higher Ka values in gene pairs with acquired differential expression in both mangrove species (Figure 4b,c; $p = 8.6 \times 10^{-4}$ and 2.8×10^{-2}, Student's *t* test), indicating that rapid evolution might have shaped neofunctionalization.

4 | DISCUSSION

4.1 | WGD facilitates adaptation

Doubling of genetic material after a whole-genome duplication is widely thought of as a driver of adaptation to novel environments. Known correlations between WGD and adaptation include the preferential retention of duplicated genes (Li et al., 2016; van de Peer et al., 2017) and the frequent coincidence of WGD events with important evolutionary transitions. For example, a WGD was identified at the stem ancestor of both eudicot and monocot clades (Jiao et al., 2014; Ming et al., 2015) and WGDs occurred in many plant lineages at the K-Pg boundary that involved drastic environmental change (Vanneste et al., 2014; van de Peer et al., 2017). Recently, Wu et al. (2019) explored the possibility that genes duplicated after WGD promote adaptation by developing new functions. They investigated 25 genomes and found that redundant gene copies independently retained after WGD had reshaped gene regulatory networks associated with cold adaptation.

Mangroves independently evolved multiple times to inhabit extreme intertidal habitats, with ambient salinity ranging from fresh water to sea water levels, an equivalent of shifting to a novel habitat, in some cases daily (Nguyen et al., 2015). We focused on two species, *Avicennia marina* and *Rhizophora apiculata*, that represent independent lineages and have experienced relatively recent WGD. We identified duplicate genes that have been retained after the WGD event and measured their transcription activity across three salt concentrations in the growth media: fresh water, intermediate salinity and salt water. We found that WGD-derived duplicate genes in both species are more likely than the rest of the genome to be differentially expressed under changing salinity conditions. Furthermore, we often see only one copy of a duplicate pair acquiring salt responsiveness. These expression changes are accompanied by elevated amino acid sequence evolution rates. Together, these observations strongly suggest that WGD events have contributed to adaptation to intertidal habitats in both *R. apiculata* and *A. marina*.

We used a combination of individual RNA pooling and two replicates to maximize confidence in gene expression estimates. In each replicate, three RNA samples were pooled to minimize variation among individual samples. The RNA pooling is a common practice among gene expression studies and is well justified based on statistical and practical considerations (Assefa et al., 2020; Kendziorski et al., 2005; Liu et al., 2011). We also examined expression estimate consistency between replicates. Direction of expression change is consistent in 86%–94% of identified DEGs across all replicate pairs. Therefore, we are confident that genes with a significant expression change have been reliably identified in our study and are suitable for evolutionary analyses.

4.2 | Neofunctionalization of WGD-duplicates expands mangrove salt adaptation

Our results suggest that neofunctionalization of retained genes may expand the range of environmental conditions that organisms can cope with. Mangroves acquire the highest biomass and grow best under medium environmental salinity (200–400 mM; Ball & Pidsley, 1995; Wang et al., 2016). Our previous study on three independent mangrove clades revealed extensive convergent amino acid composition changes relative to inland plants (He et al., 2020). These findings suggest that mangrove protein sequences have adapted to medium salinity. In this study, we found that the most enriched differential-expression combination in both mangrove species was "−1;1," indicating that gene expression was upregulated by both low and high salinity. Most "−1;1" genes (93.3% in *R. apiculata* and 97.9% in *A. marina*) are not differentially expressed between 0 and 500 mM NaCl treatments, indicating similar regulation under both conditions. Salinity dramatically fluctuates in intertidal zones as fresh and sea water mix with changing tides (Lin & Sternberg, 1993; Tomlinson, 1986). The "−1;1" genes are probably crucial for mangrove plants to cope with the stress of both low and high salt.

4.3 | A proposed model of adaptation expansion

We propose a model describing how mangroves deal with different salinity levels utilizing WGD duplicates. Amino acid sequence adaptation of proteins underlying salt tolerance led to their optimal function at medium salinity (Figure 4d). When salinity is low or high, expression of the neofunctionalized copy is upregulated to mitigate the derived activity decline. Salt tolerance can thus be maintained despite salinity change (Figure 4d). This model accounts for our observation that neofunctionalized copies of duplicate genes are upregulated at both high and no salt conditions compared to the optimal 250 mM NaCl environment. This leaves the homologous copy with stable expression to provide a baseline for normal physiological activity, while the neofunctionalized copy provides high expression plasticity. The particular genes involved differ between the species: in *R. apiculata* they are likely to code for extracellular proteins, while in *A. marina* they are often involved in plant hormone production.

4.4 | WGD expands adaptation through increasing genome plasticity

WGD is energetically costly and damages genome stability (Comai, 2005; Wagner, 2005), and thus polyploids usually occur in small populations under stable environments and subsequently undergo rediploidization. However, redundant gene copies may be less constrained by natural selection and can increase genomic plasticity in unstable environments. We indeed observe multiple instances of derived expression profile change of duplicate genes.

Single-copy genes are often involved in essential processes and are highly conserved among species (de Smet et al., 2013; Li et al., 2016). Unlike small-scale duplications, whole-genome duplication results in a dramatic immediate expansion of the whole gene regulatory network. Genes that have more interactions, such as transcription factors, are more likely to be retained after WGD (Freeling, 2009), and coordinated functional divergence of WGD-duplicates has been identified in *Arabidopsis thaliana* (de Smet et al., 2017). We also find that DEGs responding to salinity change are prevalent among WGD-duplicates in both mangrove species we studied.

Using two independently originated mangrove lineages allows us to infer convergent emergence of new expression patterns during adaptation to different salinity levels, implying that similar selection pressures operated on these mangrove genomes. Convergent evolution of mangrove genomes has previously been identified in amino acid substitutions, genome downsizing, amino acid usage bias and gene family evolution (He et al., 2020; Lyu et al., 2018; Xu, He, Guo, et al., 2017; Xu et al., 2020).

In conclusion, our study in mangroves revealed extensive neofunctionalization of salt-responsive genes retained after WGD, providing a unique example whereby retained gene duplicates increase environmental robustness, shedding new light on the mechanisms underlying salinity change tolerance in mangroves.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

S. Shi and Z.H. conceived and designed the experiments. Y.Y., J.L. and C.Z. collected materials. S.X., Y.Y. and J.L. performed the experiments. S.X., X.F. and S. Shao analysed the data. S.X., S. Shi, Z.G. and Z.H. wrote the manuscript.

DATA AVAILABILITY STATEMENT

The RNA-seq data have been deposited in the NCBI Sequence Read Archive database (Accession No. PRJNA719266) and the Genome Sequence Archive database (Accession No. PRJCA006392).

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